SULFIDE-DEPENDENT HYDROGEN EVOLUTION IN THE CYANOBACTERIUM *OSCILLATORIA LIMNETICA*

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1. Introduction

Of the oxygenic plant-type phototrophs, only cyanobacteria (blue-green algae) can use both hydrogen and sulfide as electron donors for CO_2 photoassimilation in a facultative anoxygenic photosystem I-driven reaction [1–6]. This, together with their procaryotic structure, may be relics of the long cyanobacterial evolutionary history [7].

Hydrogen evolution, endogenous or with the aid of artificial electron donors, has also been demonstrated in cyanobacteria, including those capable of facultative anoxygenic photosynthesis with sulfide as electron donor [4,8–11].

The present work shows that sulfide electrons are passed into the photosynthetic electron transport chain culminating in hydrogen evolution or CO_2 photoassimilation in a photosystem I driven reaction in *Oscillatoria limnetica* cells. This occurs when the cells are induced to anoxygenic photosynthesis in the presence of sulfide.

2. Materials and methods

Oscillatoria limnetica cells [1] were grown aerobically or anaerobically as in [6].

Hydrogen evolution was determined in cells, washed and resuspended (2–5 μg chlorophyll *a*/ml) in the growth medium in [6] but lacking Na_2CO_3 and in the presence of 10 μM [3-(3,4-dichlorophenyl)-1,1-dimethyl urea] (DCMU) and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.5). Cell-free systems were obtained by sonication (Branson, model S110) of the washed-cell suspension for 30 s at 4°C. Serum bottles, 10 ml, containing 1.5 ml of either intact or broken-cell suspensions were sealed with sleeve rubber stoppers, flushed with argon for 15 min, and incubated at 35°C for different times with shaking as in [4]. All additions and withdrawals were made with gas-tight syringes. The reaction was terminated by the addition of 0.5 ml of 1 N $(CH_3COO)_2Zn$; 0.5 ml of the gas phase was then injected into a Packard model 427 gas chromatograph equipped with a thermal conductivity detector and a 180 × 0.6 cm glass column of Molecular Sieve 5A (80 × 100 mesh). Filament temperature was 400°C and argon served as carrier gas at a flow rate of 45 ml/min. The output signal was recorded with a Goertz model RE541 Servogor recorder. The retention time for hydrogen was 50 s. Concentrations were calculated by reference to hydrogen standards.

For determination of CO_2 photoassimilation the system was first flushed with argon and then injected with $Na_2{}^{14}CO_3$ to yield final conc. 14.1 mM (spec. act. 0.85 μCi/ml). After 30 min a gas sample was withdrawn for measurement of H_2 evolution and the CO_2 photoassimilation was determined in 1.0 ml of the suspension as in [4]. Chlorophyll was assayed as in [12].

Methylviologen, *N'*,*N'*-dicyclohexylcarbodiimide (DCCD) and Hepes were obtained from Sigma. DCMU, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were obtained from DuPont. Disalicylidenepropanediamine (DSPD) and 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) were a gift from Professor M. Avron (Weizmann Institute of Science, Rehovot).

3. Results and discussion

Aerobically-grown *O. limnetica* cells evolve hydrogen in the dark in the presence of the artificial electron donor, methylviologen (Na-dithionite reduced) (fig.1). The observed rate for intact cells or cell-free systems is 20–25 μmol/mg chlorophyll *a*/h and 10–15 μmol/mg chlorophyll *a*/h, respectively. A similar capacity for hydrogen evolution has been recently observed in *Aphanothece halophytica* [4] which like *O. limnetica* is a non-heterocystous cyanobacterium.

Both *A. halophytica* and *O. limnetica* are among the many cyanobacteria in which the capacity to use sulfide as electron donor for CO_2 photoassimilation has been demonstrated [3]. Sulfide inhibits oxygenic photosynthesis in aerobically grown *O. limnetica* cells [6]. However, when these cells are preincubated for 3 h in the presence of light and sulfide (with or without DCMU) synthesis of new proteins is induced allowing photosystem I-driven anoxygenic photosynthesis, with sulfide as electron donor, to take place ([6], fig.1). After induction, these cells can even grow photoautotrophically under anaerobic conditions with a doubling time of 48 h, similar to the aerobic one [6].

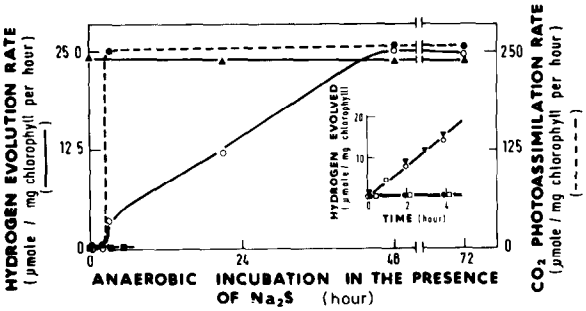

Fig.1. Hydrogen evolution and anoxygenic CO_2 photoassimilation by *O. limnetica* cells after various periods of anaerobic incubation in the presence of sulfide. Day 4 aerobically grown cells were resuspended (2–3 μg chlorophyll *a*/ml) in anaerobic growth medium containing 2.2 mM Na_2S and 10 μM DCMU and incubated under anaerobic growth conditions [6] in the absence (○) or in the presence (■) of chloramphenicol (10 μg/ml). For determination of H_2 evolution rate cells samples were washed and resuspended (2–4 μg chlorophyll *a*/ml) in fresh anaerobic growth medium lacking Na_2CO_3 but containing 10 μM DCMU, 10 mM Hepes and 3.0 mM Na_2S. For determination of H_2 evolution from reduced methylviologen (▲), Na_2S was omitted and methylviologen (5 mM) and Na-dithionite (20 mM) were added. The cells were incubated under argon gas phase at 35°C in the light (2×10^4 erg/cm^2/s) and the evolved hydrogen determined. For determination of CO_2 photoassimilation (●) 14.1 mM $Na_2{}^{14}CO_3$ (0.85 μCi/ml) was added to the appropriate reaction mixtures.

Inset: Sulfide and light requirement of hydrogen evolution in *O. limnetica*. Cells preincubated under anaerobic growth conditions, in the presence of sulfide and DCMU for 3 h (induction period) were prepared and incubated for hydrogen evolution assays as in fig.1 (○) or with the following modifications: incubation in the dark (□); sulfide omitted (◐); gas phase nitrogen (▼).

The 3 h preincubation of *O. limnetica* cells in the presence of sulfide and light induces another activity, both photosystem I and sulfide-dependent, i.e., hydrogen evolution, detected when CO_2 is omitted from the reaction mixture (fig.1 and inset). The presence of chloramphenicol (fig.1) or the omission of either sulfide or light during the period of preincubation (not shown) prevents both the induction of anoxygenic photosynthesis [6] and the evolution of hydrogen. Even after the induction of anoxygenic photosynthesis, evolution of hydrogen will not take place in the dark or in the absence of sulfide (fig.1, inset).

The rate of the sulfide-dependent hydrogen evolution in the induced cells is 3.7 μmol/mg chlorophyll *a*/h; it is 7-times lower than the rate observed in the presence of methylviologen in such cells or in non-induced ones (fig.1). Hence it is suggested that the new enzymes synthesized during the induction period are not involved in the hydrogenase activity itself but rather in the mechanism of sulfide penetration into the cells and/or sulfide oxidation. The slow rate of sulfide-dependent hydrogen evolution as compared to the methylviologen-requiring process indicates that there is a rate-limiting step in the electron flow from sulfide to hydrogen ions. However, when the cells are further incubated in the presence of light and sulfide, the rate of the sulfide-dependent hydrogen evolution slowly increases until a maximal rate similar to the methylviologen-dependent one is obtained after 2 days (fig.1). This is the period necessary for the doubling of *O. limnetica* during anaerobic growth. It is suggested that during this period dilution and synthesis of factors in the growing cells alleviate the rate-limiting step in the

Table 1
The effect of the addition of various inhibitors as well as carbonate on sulfide-dependent hydrogen evolution by *O. limnetica*

Compounds added (M)		H_2 evolution rate (μmol/mg chl *a*/h)	% control
1. None		29.2	100
2. DSPD	10^{-3}	0	0
	10^{-4}	31.5	108
3. DCCD	5×10^{-5}	35.1	120
	5×10^{-6}	31.4	108
4. FCCP	2×10^{-5}	40.0	137
	2×10^{-6}	30.1	103
5. SF6847	10^{-5}	47.7	163
	10^{-6}	40.1	137
6. CCCP	10^{-5}	50.2	172
	10^{-6}	36.4	125
7. Na_2CO_3	14.1×10^{-3}	0	0
8. Na_2CO_3 FCCP	14.1×10^{-3} 10^{-5}	33.9	116

The reaction mixture contained 2–5 μg chlorophyll *a*/ml of day 3 anaerobically grown cells, growth medium [6] lacking Na_2CO_3 but containing 3 mM Na_2S, 10 μM DCMU and 10 mM Hepes (pH 7.5). All compounds added were injected into the reaction mixtures after argon flushing

sulfide-dependent hydrogen evolution. Note, however, that the maximal rate of sulfide-dependent hydrogen evolution is 10-times lower than the maximal rate of sulfide-dependent CO_2 photoassimilation (fig.1). Increased hydrogenase activity during anaerobic growth in a hydrogen gas phase has been demonstrated in *Anabaena cylindrica* and *Nostoc muscorum* [8]. However, in this case, induced synthesis of the hydrogenase has been implicated.

The enzyme involved in the sulfide-dependent H_2 evolution of *O. limnetica* appears to be a hydrogenase rather than a nitrogenase since the presence of nitrogen which inhibits nitrogenase-dependent hydrogen evolution [13] is ineffective (fig.1, inset); the uncouplers FCCP, CCCP and SF 6847 [14] which inhibit the ATP-requiring nitrogenase [9] accelerate the sulfide-dependent hydrogen evolution (table 1) and DCCD which inhibits ATPase-dependent phosphorylation has no effect on this hydrogen evolution (table 1).

The photosynthetic electron transport inhibitor DSPD which inhibits ferredoxin activity inhibits the sulfide and photosystem I-dependent hydrogen evolution (table 1). Upon addition of CO_2 into the reaction mixture the maximal rate of the anoxygenic CO_2 photoassimilation is resumed and at the same time there is severe inhibition of sulfide-dependent hydrogen evolution (table 1). It is therefore suggested that:

(i) Sulfide donates electrons to the electron transport chain at a site preceding photosystem I.
(ii) In the absence of CO_2 the electrons are expelled by hydrogen evolution at or after the ferredoxin site.
(iii) In the presence of CO_2 the electrons are channelled into CO_2 photoassimilation.

Indeed, the addition of FCCP to the CO_2 photoassimilating system inhibits the latter's activity, hydrogen evolution being resumed and even accelerated. This enhancement also suggests that electron transport from sulfide to hydrogen ions is coupled to photophosphorylation.

The *Oscillatoria limnetica* photosynthetic system, in which photosynthetic electron transport may be rapidly channelled to the evolution of hydrogen with sulfide as the cell's innate inorganic electron donor, may serve as a model system for testing the feasibility of such biological energy sources.

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